(54) Title: METHOD OF PREPARING OLIGONUCLEOTIDE PROBES OR PRIMERS, VECTOR THEREFOR AND USE THEREOF

(57) Abstract

    A method of preparing a nucleic acid sequence capable of hybridizing to a target DNA sequence without requiring knowledge of this DNA sequence comprises the steps of ligating or otherwise linking a DNA fragment related to the target DNA sequence to a nucleotide sequence containing the recognition motif of an asymmetrically cleaving restriction enzyme, and subjecting the construct to the restriction enzyme to thereby cleave the construct within the target-related DNA fragment part thereof. A vector therefor comprises a site for insertion of the target-related DNA fragment, and a recognition sequence for an asymmetrically cleaving restriction enzyme or enzymes on one or both sides of said insertion site.

METHOD OF PREPARING OLIGONUCLEOTIDE PROBES OR PRIMERS, VECTOR THEREFOR AND USE THEREOF

FIELD OF THE INVENTION

5   The present invention relates to the preparation of nucleic acid probes and primers as well as vectors therefor.

BACKGROUND OF THE INVENTION

The polymerase chain reaction, or PCR, provides a
10   highly efficient method of isolating a desired gene sequence(s) from different DNA samples. One general requirement is that sequence information is available from both ends of the fragment to be amplified in order to synthesize specific amplification primers. The expense of
15   sequencing DNA and then chemically synthesizing primers still limits the scope of many applications. In many investigations it would therefore be most helpful if the steps of DNA sequencing and oligonucleotide synthesis could be avoided entirely before genomic fragments are amplified
20   by PCR. This is true for instance for the large programs designed to analyze the degree of polymorphism of specific DNA segments in individuals in a population, in order to identify polymorphic markers in the human or other genomes. Such genetic markers are required for mapping genetic
25   disease and to establish or extend genetic linkage maps for a variety of organisms.

One approach which partially overcomes the above problem is the use of relatively nonspecific amplification with a limited set of primers under nonstringent
30   conditions, so called RAPD markers. These markers serve as a means to establish relatively random polymorphic markers, analyzable by PCR without requiring DNA sequencing and chemical oligonucleotide synthesis of specific primers.

SUMMARY OF THE INVENTION

35   The present invention provides a different strategy to overcome the deficiences of the prior art methods and permits enzymatic synthesis of specific amplification primers, ligation probes or other probes of known structure

but partially unknown sequence without access to clone specific DNA sequence information.

In one aspect, the invention provides a vector for the preparation of a nucleic acid sequence of known structure but partially unknown sequence, capable of hybridizing to a target DNA sequence without requiring knowledge of this DNA sequence, which vector comprises a site for the insertion of a DNA fragment related to the target DNA sequence, and a recognition sequence for an asymmetrically cleaving restriction enzyme or enzymes on one or both sides of said insertion site.

In another aspect, the invention provides a method of preparing a nucleic acid sequence of known structure but partially unknown sequence, capable of hybridizing to a target DNA sequence without requiring knowledge of this DNA sequence, which method comprises the steps of ligating or otherwise linking a DNA fragment related to the target sequence to a nucleotide sequence containing the recognition motif of an asymmetrically cleaving restriction enzyme, and subjecting the construct to the restriction enzyme to cleave the construct within the DNA fragment part thereof.

In still another aspect, the invention provides a method for amplifying a DNA fragment without requiring knowledge of the DNA sequence of the fragment by using enzyme-synthesized primers prepared according the above aspect of the invention.

In yet another aspect, the invention provides a method for constructing probes for target-dependent ligation reactions.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description thereof which includes an illustrative example of the practice of the invention.

The invention will be described in more detail below with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B are a schematic representation, in the form of four parts A-D, of a strategy to derive amplification primers enzymatically. Part A shows the construction of a specialized cloning vector, part B shows the cloning of an AluI fragment into the vector, part C shows enzymatic synthesis of amplification primers, and part D shows amplification of sequences corresponding to those cloned in the vector using primers derived from the amplified insert of the clone. The recognition sequences of the restriction enzymes GsuI and AluI are highlighted in the figures.

Fig. 2 is a schematic representation of an immobilized vector containing a GsuI site, the cleavage thereof to prepare a circularizable probe, and the use of the latter for the capture and subsequent release of single-stranded circular target molecules.

Fig. 3 is a photograph of an autoradiograph of a comparison of amplification products obtained by PCR using sets of primers derived by solid-phase chemical synthesis or enzymatically. For an AluI fragment of a known DNA sequence, specific 16-mer primers that were synthesized by standard solid phase chemistry, were compared with similarly constructed 38-mer primers of the same 16-base sequence at the 3'-end, and with an enzymatically synthesized primer pair, expected to have the same 38-mer sequence as the chemically synthesized oligonucleotides of this size.

DETAILED DESCRIPTION OF THE INVENTION

A restriction endonuclease, or restriction enzyme, is an enzyme capable of recognizing a specific DNA sequence (usually four to eight nucleotides in sequence) and cleaving the DNA, thereby creating double-stranded breaks. The restriction enzymes are grouped into four classes, type I, II, III and IV, on the basis of their subunit structure, cofactor requirements, substrate specificity and several other features. Type I enzymes cleave the substrate DNA almost at random at a great distance from the recognition

site. Type II enzymes, exemplified by EcoRI, cleave within
the recognition site or a few nucleotides away. Both type
III and type IV enzymes cleave the target DNA sequence some
considerable distance away from the recognition site and
they also have in common the property of never cleaving the
DNA to completion. Type IV enzymes differ from the type III
enzymes in that they, similar to the type II restriction
enzymes, are not dependent upon ATP. The type III and type
IV restriction enzymes are commonly called asymmetrically
cleaving enzymes. Exemplary of type IV restriction enzymes
are GsuI (Janulaitis et al., Nucl. Acid Res. 1989;
17(14):1989), with the recognition motif 5'CTGGAG3' and
cleaving 16 bases downstream of this motif; Eco57I, with
the recognition sequence 5'CTGGTG3' and cleaving 16 bases
downstream of this motif; MmeI (Boyd et al., Nucl. Acid
Res. 1986; 17(14):5255-5274, with a 6-base recognition
motif, and which cleaves the target DNA 22 bases downstream
of this sequence. Restriction enzyme BpmI (available from
New England Biolabs, U.S.A.) has the same recognition
sequence and cleaves in the same way as GsuI above. There
are also type III or IV enzymes cleaving the target DNA
even farther from the recognition sequence. Examples of
such enzymes include Eco15 and HinP15I cleaving 25 or 26
bases downstream of the recognition sequence.

     According to the present invention, the property of
type III or IV restriction enzymes to cleave
asymmetrically, i.e. to cleave a DNA substrate at a
distance from the recognition sequence, is utilized to
prepare probes or primers of a defined size capable of
hybridizing to a target DNA sequence without a requirement
to know this DNA sequence.

     In one embodiment of the invention, amplification
primers are prepared which may then be used, optionally in
combination with a standard vector sequence-derived
oligonucleotide, for amplification to large copy numbers,
starting from a genomic DNA sample.

     Thus, defined size amplification primers may be
produced enzymatically through digestion inside the cloned

fragments in amplification products by locating a
recognition sequence for an asymmetrically cleaving
restriction enzyme on both sides of the cloning site. The
digestion is independent of the DNA sequence of the cloned
5   fragment, and the cleavage products consisting of the
original amplification primers having linked thereto a
respective part of the cloned DNA fragment may be used to
amplify sequences corresponding to the cloned DNA fragment
from c   er DNA samples. Such a procedure for producing
10   amplification primers will now be described in more detail.
        With reference to Fig. 1A, part A, a plasmid vector is
cleaved at a desired site, and an oligonucleotide duplex,
or linker, containing the recognition site for an
asymmetrically cleaving restriction enzyme, in the figure
15   by way of example the recognition motif for GsuI, is
ligated to each vector fragment end. Both linkers lack 5'-
phosphate groups to prevent them from ligating to
themselves. Ligation of linkers to the restriction fragment
ends does not recreate the restriction site, so that over
20   time more and more restriction ends are modified with
linker dimers. The vector produced is then isolated, e.g.
by gel purification, from remaining circular vector
molecules and used for molecular cloning of DNA fragments.
This is schematically illustrated in Fig. 1A, part B which
25   shows the construction of a recombinant molecule by
ligating a blunt end AluI-cleaved DNA-fragment into the
vector.
        In order to produce amplification primers, individual
clones are amplified using a single primer corresponding to
30   one of the linker oligonucleotides and with the 5'-end
protected against λ exonuclease digestion, e.g. by a biotin
residue at the 5' end. The resulting amplification products
consist in the cloned AluI-fragment surrounded by vector-
derived sequences representing the oligonucleotide dimer
35   ligated to the vector as described above. With reference to
part C of Fig. 1A, the 6-base motif, 5'CTGGAG3',
immediately flanking the insert cloned into the vector,
represents the recognition sequence for the restriction

enzyme GsuI. As mentioned above, this enzyme cleaves the
DNA molecule 16 bases downstream of this motif, inside the
fragment cloned into the vector. After digesting the
amplification products with this enzyme, the fragments
5   obtained are separated by gel electrophoresis. Thereby the
fragments derived from both ends of the amplification
product may be quantitated and isolated from remaining
undigested or partially digested molecules. Finally, any
DNA strands that have 5' phosphate groups are removed by
10   digestion with the enzyme λ exonuclease, whereas the
extension products containing 5' biotin are insensitive to
the exonuclease over a wide range of nuclease
concentrations. In this manner, pairs of single-stranded
amplification primers are obtained, which consist of a 5'
15   end part, derived from the linker sequence that was added
to the cloning vector by linker ligation, and a 3' end
part, derived from ends of the inserts cloned into the
vector. In the illustrated case (Fig. 1A, part C) there is
located immediately upstream of these 16 bases in the
20   primers a dinucleotide, AG, representing the last two
positions in the recognition sequence for the enzyme GsuI.
These positions are also known to be present in the genomic
DNA from which the cloned fragment was derived since these
fragments were derived by digestion with the blunt end
25   generating restriction enzyme AluI (Fig. 1A, part C). Each
one of the enzyme-synthesized primers therefore has a
number (here 18) of bases (the 3' end bases plus two of the
5' end bases) that are complementary to the genomic
sequence at each side of the cloned fragment, and a number
30   of bases derived from the vector.

        The above enzymatically synthesized primers may be
used for PCR amplification of a fragment obtained by AluI
digestion of chromosomal DNA as schematically illustrated
in Fig. 1B, part D. First, the primers are used in a number
35   of, say 5, amplification cycles, when necessary using a
long annealing time to compensate for relatively low
concentration of enzyme-synthesized primers. In these first
cycles, the desired DNA fragments are obtained, flanked at

either end by the vector sequence. It is then no longer
necessary to use the enzymatically synthesized primers, but
the further amplification of the fragments to large numbers
may conveniently be performed with the "standard" vector-
5   derived primer, i.e one of the linker primers or a variant
thereof.

In a variant of the above procedure, a cloning vector
is prepared which contains the two recognition sites for
the asymmetrically cleaving enzyme immediately adjacent
10   each other but in opposite orientations and together
containing the recognition site for a bluntly cleaving
restriction enzyme, such that the enzyme may cleave right
between the two sites. This permits the cloning vector
containing the two recognition sites for the asymmetrically
15   cleaving enzyme to be amplified prior to blunt cleavage
between the sites, phosphatase treatment to prevent self-
ligation of the free vector ends, and insertion and
ligation of the target DNA sequence to be subsequently
cleaved by the asymmetrically cleaving enzyme. As an
20   example may be mentioned two juxtaposed but opposed GsuI
(= BpmI) sites which can be bluntly cleaved between them
b the restriction enzyme Ecl136II (New England Biolabs,
U.S.A.)

As appears from the above, the steps of the present
25   pr dure are simple and may easily be performed for a
lai number of fragments in parallel, permitting the
development of large sets of genetic markers. If desired,
successful markers may be sequenced and converted to
regular chemically synthesized PCR-based primers, but it is
30   also possible to continue using enzyme-synthesized primers.

Enzymatically synthesized amplification primers,
constructed as described above, can also provide highly
specific and sensitive detection reagents. Thus, in another
embodiment of the invention, amplification primers suitable
35   to detect individual organisms in complex mixtures by PCR
may be obtained by restriction digesting DNA preparations,
cloning the fragments into the specialized vector described
above, and isolating individual clones in order to generate

a desired number of pairs of amplification primers. These
amplification primers may thus be developed and tested
without any requirement for access to DNA samples from
isolated organisms or for DNA sequence information from

5   these.

In order to avoid the risk of contamination by another
fragment, amplifiable with the same standard primer,
precautions such as the inclusion of a ribose in the 3'
position of the standard primer, rendering amplification

10   products cleavable by alkali, may be contemplated.

It is readily seen that the same DNA fragment may be
cloned into two different vectors with different linkers,
or "standard primers", permitting so-called nested PCR to
be performed. This is possible by using different flanking

15   sites for enzymes that cleave at variable distances
internal to the cloned fragment. In nested PCR, a number of
cycles are conducted with a first set of primers, whereupon
a number of cycles are run with a second set of primers
hybridizing internally of the first primer set. This

20   increases the efficiency and selectivity of the PCR
process.

As is readily realized, it is essential in the
preparation of the synthesized primers to isolate the
desired end fragments obtained after cleavage with the

25   asymmetrically cleaving enzyme from any remaining partially
digested or undigested amplification products as incomplete
digestion products may give rise to false positives in the
subsequent amplification, when using the enzymatically
derived primers. In the preparation procedure described

30   above, gel electrophoresis is used to isolate the desired
fragments. However, preparative gel electrophoresis is
relatively cumbersome and may also fail to adequately
resolve end fragments and incomplete digestion products. An
alternative means of removing middle piece-containing

35   fragments away from the desired end fragments is described
below.

Despite the fact that the middle segments are of
unknown sequence composition, end fragments may be

efficiently isolated by amplifying the cloned fragments in two separate reactions as follows:

In each of the two amplifications of the cloned inserts, one or the other amplification primer is
5   biotinylated, permitting the amplified molecules to be isolated on a solid support, such as streptavidin-coated combs, e.g. of the type described in WO 94/11529, or paramagnetic particles. Other means of immobilization including DNA ligation may also be used. After adequate
10   washes, one or the other of the end fragments are released from the rest of the amplified molecules by selectively digesting the asymmetric restriction enzyme site located at the end remote from the support. This selective digestion may be achieved by using vectors with recognition sequences
15   for two different asymmetric restriction enzyme sites, e.g. type IV enzymes, on each side of the cloned inserts and cleaving the immobilized, amplified molecules in two separate reactions. Examples of such pairs of type IV restriction enzymes are GsuI and Eco57I.
20   Alternatively, vectors with a single restriction site on both sides of the insert may be used if the biotinylated amplification primers are designed such that they are mismatched to the vector in e.g. positions 5 and 6 in from the 3' end of the primer, corresponding to the first 2
25   bases of the 6 base recognition sequence of a type IV restriction enzyme. Other sequence alterations are, of course, also possible, preserving the ability of the primers to amplify the cloned fragment but destroying the recognition sequence for one or the other of the sites. In
30   this strategy, the two amplification products may be pooled before immobilization on the supports and restriction cleavage. After cleavage, released fragments are treated with λ-exonuclease to obtain single stranded probes, and may then be directly used as enzymatic amplification
35   primers.

The enzymatic primer synthesis approach of the invention may also be used for producing circularizable probes. The preparation and use of circularizable probes

(so-called "padlock" probes) have been described by
Landegren et al., Science 265, 2085-2088 (1994). Such
probes consist of two target-complementary segments,
connected by a linker sequence. Upon recognition of a
5   specific nucleic acid molecule, the ends of the probe are
joined through the action of a ligase, creating circular
DNA molecules catenated to the target sequence. These
probes provide highly specific detection with minimal
background.

10   In accordance with another embodiment of the
invention, such a circularizable probe may be prepared by
cloning a DNA sequence into a specially designed vector,
and the vector may then be used as a circularizable probe
without the use of DNA sequence information from the cloned
15   fragment, or the use of specific oligonucleotides. A large
set of such clones may be processed in parallel for e.g.
subtractive comparisons between cDNA populations. For
example, library to library comparisons may be made between
for instance a library of all genes expressed in a given
20   tissue and on the other hand all sequences located in a
particular region of the genome, from yeast artificial
chromosomes, subcloned in a circular single stranded
plasmid library. This is, for example, useful when a
disease gene has been located to a region by linkage
25   analysis and it is known to be expressed in a particular
tissue.

            The necessary vector may be prepared by ligating
oligonucleotides to a restriction digested plasmid to
generate, on one hand, a recognition motif for an
30   asymmetrically cleaving enzyme, such as GsuI, and, on the
other hand, a nearby situated rare restriction site, such
as NotI. One way of constructing the vector is by inserting
two oligonucleotide dimers to two different ends, generated
by digestion with two restriction enzymes.

35   Recombinant molecules are then produced by ligating
blunt end DNA fragments, e.g. generated by AluI digestion,
to the vector. The constructs obtained are transformed into

bacteria and single-stranded recombinant molecules are
generated.

These circular single-stranded molecules are then
immobilized in patches on a solid phase, e.g. a membrane,
5   as schematically illustrated in Fig. 2. A primer is
hybridized to the vector molecule and extended using a DNA
polymerase, rendering the recognition sequence for the
asymmetrically cleaving enzyme double stranded and
permitting cleavage of the insert. After digestion of the
10   molecules with the enzyme, the fragments of extended
molecules are removed by denaturing washes.

The immobilized circularizable probes obtained may be
used for trapping target molecules, as is also illustrated
in Fig. 2. To this end, a population of recombinant clones
15   prepared as above is turned into single-stranded circular
molecules. For comparisons between directionally cloned
cDNA populations, this library, should, of course, be of
the opposite polarity to the above construct. Further, the
vector must not have the same rare-cutter enzyme site or at
20   least not the same polarity of the sequence surrounding the
recognition sequence. These molecules are now exposed to
the immobilized probes and trapped by ligation, followed by
stringent washes.

An oligonucleotide is then annealed to the region of
25   the rare-cutter side on the capture vectors, and these are
linearized by digestion with the rare cutter enzyme.
Trapped clones are released by denaturing washes, repaired,
and transformed into bacteria for molecular cloning.

Since the above described approach is based on an
30   intramolecular reaction (ligation), large numbers of clones
may be treated and then applied as capture probes in the
same reaction. In contrast, e.g. PCR will only permit a
handful of reactions to be performed in the same reaction
due to the problem of nonspecific crosstalk between
35   primers.

In the following, the invention will be illustrated by
a non-limiting example.

EXAMPLE
Chemical oligonucleotide synthesis

The following oligodeoxynucleotides were synthesized
on an Applied Biosystems 394 DNA/RNA synthesizer (Applied
5   Biosystems Inc., USA):
- Omni1: 5' AATTG ACCGT TAGCA ACTGG AG 3'
- Omni2: 5' CTCCA GTTGC TAACG GTC 3'
- BOmni1: Omni1 above syntesized with the addition of a 5'
biotin residue.
10   (In the sequences for Omni1 and Omni2 the recognition
sequence for the restriction enzyme GsuI has been
underlined.)
- Control primer A1: 5' AATTG ACCGT TAGCA ACTGG AGCTC AGCAG
CCCGT GAT 3'
15   - Control primer AM1: 5' CTC AGCAG CCCGT GAT 3'
- Control primer A2: 5' AATTG ACCGT TAGCA ACTGG AGCTT CCAGA
GTCAG ATC 3'
- Control primer AM2: 5' CTTCC AGAGT CAGAT C 3'.

Construction of the cloning vector pRIMER

20   Plasmid pBLUESCRIPT M13 was digested with EcoRI and,
in the same reaction, the oligonucleotide duplex Omni1 and
Omni2 were ligated to the restriction fragment ends (Fig.
1A, part A). 20 U of EcoRI were added to 5 µg of the
plasmid in a final volume of 10 µl containing 10 mM Tris-
25   HCl, pH 7.5, 10 mM MgCl$_2$, 50 mM potassium acetate, and 5 mM
ATP. After 3 hours at 37°C, 2.5 U of T4 DNA ligase were
added with oligonucleotides Omni1 and Omni2 at 25 µM each.
The reaction was left at 14°C over-night. Linearized vector
molecules with added linkers were gel purified in a 1.5%
30   NuSieve GTG agarose gel (Nalgene), and used as a cloning
vector.

Cloning of Alu-digested fragments and screening for positive clones

AluI-digested fragments from the human ameliogenin
35   gene located on the X chromosome were ligated into the
above vector and introduced in strain DH5 of E. coli (Fig.
1A, part B). Individual bacterial clones were transferred
with a toothpick from the agar plate to 100 µl of water in

an Eppendorf tube, the samples were boiled for 5 minutes,
and then chilled on ice. Cell debris was pelleted in a
short centrifugation and one µl of the supernatant was used
as a source of template in a 30 µl PCR with the single

5   primer BOmni1 at 1 µM. 10 µl aliquots of the PCR samples
were separated in a 1.5% agarose gel to quantitate and
determine the size of the amplification products before the
inserts were used for synthesis of amplification primers.

Enzymatic synthesis of specific amplification primers

10   In order to prepare amplification primers (Fig. 1A,
part C), 2 µg of inserts that had been amplified with
BOmni1 were gel purified in 2% NuSieve GTG agarose (Nalgen)
and digested with 8 units of GsuI (Fermentas, Vilnius,
Lithuania) in 15 µl of 100 mM NaCl, 50 mM Tris-HCl, pH 7.9,

15   10 mM $MgCl_2$, 1 mM dithiothreitol, and BSA at 0.1 µg/µl for
3 hours at 37°C.

The digestion products were separated by
electrophoresis in a 2% agarose gel. End fragments released
from the amplification products by enzymatic digestion were

20   isolated from the gel, taking care not to contaminate the
material with undigested material.

Isolated fragments were treated with λ exonuclease
(Life Technologies) destroying DNA strands with 5'-
phosphate groups but sparing strands with a biotin residue

25   at the 5'-position. The reactions were performed in a final
volume of 10 µl, containing approximately 200 ng of end
fragments, 50 ng/µl BSA, 2 units of λ exonuclease, 67 mM
glycine-KOH, pH 9.4, 2.5 mM $MgCl_2$ for 1 hour at 37°C. The
DNA was preheated for 5 minutes at 94°C before the

30   reaction. Remaining 5'-biotinylated DNA strands were used
as amplification primers.

Amplification using enzyme-synthesized primers

Enzymatically synthesized primers were used at a
concentration of 0.05 µM, together with oligonucleotide

35   Omni1 at 1 µM, for amplification of human genomic DNA
samples (Fig. 1B, part D). During the first 5 amplification
cycles, annealing times were set at 10 minutes followed by
a regular amplification protocol. Since the melting

temperatures are not known for the template-specific 3'-
ends of the amplification primers, two different different
amplification programs were tried for each primer pair. The
complete amplification program was as follows: 5 cycles of
5   94°C for 1 minute, annealing at 40°C or 55°C for 5-15
minutes, and 72°C for 1 minute, followed by 30 cycles of 94
°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute.

        The autoradiograph in Fig. 3 illustrates agarose gel
electrophoresis of the results of amplification reactions
10   using either chemically synthesized, 16 bases long primers
(Am1 and Am2), lane 2 and lane 3 (without template); the
same 16 bases with additionally 22 bases at the 5' ends
identical to Omni1 (A1 and A2), lane 4 and lane 5 (without
template); amplification with BOmni1 from the plasmid clone
15   used to generate the primers, lane 6 and lane 7 (without
template); or enzyme-synthesized primers for a 200 bases
long AluI fragment of known DNA sequence, assumed to be
identical in sequence to oligonucleotides A1 and A2, lane 8
and lane 9 (without template). Lanes 1 and 10 are 100 bp
20   ladders. As appears from the autoradiograph, all three
pairs of oligonucleotides, when applied to human genomic
DNA samples, gave rise to amplification products of
approximately the same size. This is the size expected for
these primers. In the absence of added template DNA, no
25   amplification products were observed, affirming that the
amplification products did not represent copies of
contaminating undigested amplified clone inserts.

                    Identification of polymorphic sequences

        The amplification primer Omni1 was kinased with ^{32}P in
30   a 30-minute reaction at 37°C in 50 mM Tris-HCl, pH 7.5, 50
mM KCl, 10 mM MgCl$_2$, and 0.5 ICI γ^{32}P ATP. The primer was
used for amplification of genomic DNA samples, followed by
separation of the products by 6% polyacrylamide denaturing
gel electrophoresis. Size variation among amplification
35   products was revealed by autoradiography.

CLAIMS

1.   A method of preparing a nucleic acid sequence capable of hybridizing to a target DNA sequence without requiring knowledge of this DNA sequence, **characterized** in that the method comprises the steps of ligating or otherwise linking a DNA fragment related to the target DNA sequence to a nucleotide sequence containing the recognition motif of an asymmetrically cleaving restriction enzyme, and subjecting the construct to the restriction enzyme to thereby cleave the construct within the target-related DNA fragment part thereof.

2.   The method according to claim 1, **characterized** in that the method comprises linking said nucleotide sequence to each end of a linearized circular vector, inserting and ligating said target-related DNA fragment into the vector, optionally multiplying said vector, and cleaving the assembly of said two nucleotide sequences and said DNA fragment from both ends within the DNA fragment by said asymmetrically cleaving restriction enzyme.

3.   The method according to claim 2, **characterized** in that the method comprises the step of amplifying the assembly of said two nucleotide sequences and said DNA fragment prior to the cleavage step.

4.   The method according to claim 3, **characterized** in that said amplification is performed by enzymatic amplification using a primer hybridizing to said nucleotide sequence.

5.   The method according to claim 4, **characterized** in that said primer at the 5' end thereof is protected against λ exonuclease digestion, for example by biotinylation, and that the unprotected strands obtained in the amplification process are subjected to λ exonuclease digestion.

6.   The method according to claim 1, **characterized** in that
the method comprises linking said nucleotide sequence
containing the recognition motif of an asymmetrically
cleaving restriction enzyme to one end of a linearized
5   nucleic acid plasmid vector, inserting and ligating said
target-related DNA fragment into the vector, and cleaving
the vector within said DNA fragment by said restriction
enzyme to thereby form a circularizable probe.

10   7.   The method according to claim 6, **characterized** in that
the method further comprises providing a rare restriction
site near said restriction enzyme recognition motif.

8.   The method according to any one of claims 1 to 7,
15   **characterized** in that after said cleavage, the desired
hybridizing nucleotide sequence or sequences are isolated
by gel purification, preferably gel electrophoresis.

9.   The method according to any one of claims 3 to 8,
20   **characterized** by immobilizing the amplified fragments to a
solid support prior to performing the cleavage step.

10.   The method according to claim 9, **characterized** by
providing a recognition sequence for an asymmetrically
25   cleaving restriction enzyme on each side of the target-
related DNA fragment, performing two amplification
reactions to obtain two amplified fragments that bind to
the solid support, and selectively cleaving each amplified
fragment at the asymmetric enzyme restriction site located
30   at the end remote from the support.

11.   The method according to claim 10, **characterized** by
providing different asymmetrically cleaving restriction
enzyme sites on each side of the target-related DNA
35   fragment, and cleaving the immobilized amplified fragments
in two separate reactions.

12. A vector for the preparation of a nucleic acid sequence of known structure but partially unknown sequence capable of hybridizing to a target DNA sequence without requiring knowledge of this DNA sequence, **characterized** in

5   that the vector comprises a site for the insertion of a DNA fragment related to the target DNA sequence, and a recognition sequence for an asymmetrically cleaving restriction enzyme or enzymes on one or both sides of said insertion site.

10

13. The vector according to claim 12, **characterized** in that, after insertion of said DNA fragment, the vector may be rendered single-stranded and is capable of forming a circularizable probe after cleavage by said asymmetrically

15 cleaving restriction enzyme.

14. The vector according to claim 13, **characterized** in that it further comprises a rare restriction site near said recognition sequence.

20

15. The vector according to claim 13 or 14, **characterized** in that it has said DNA fragment inserted into said site and optionally is immobilized to a solid support, such as a membrane.

25

16. A method for amplifying a DNA fragment without requiring knowledge of the DNA sequence of the fragment, **characterized** by using as extension primers enzyme-synthesized primers prepared according to any one of claims

30 1 to 5.

17. The method according to claim 16, **characterized** by performing a first number of amplification cycles by said enzyme-synthesized primers, and then performing a second

35 number of amplification cycles by chemically synthesized primers capable of hybridizing to said nucleotide sequence linked to the linearized vector ends.
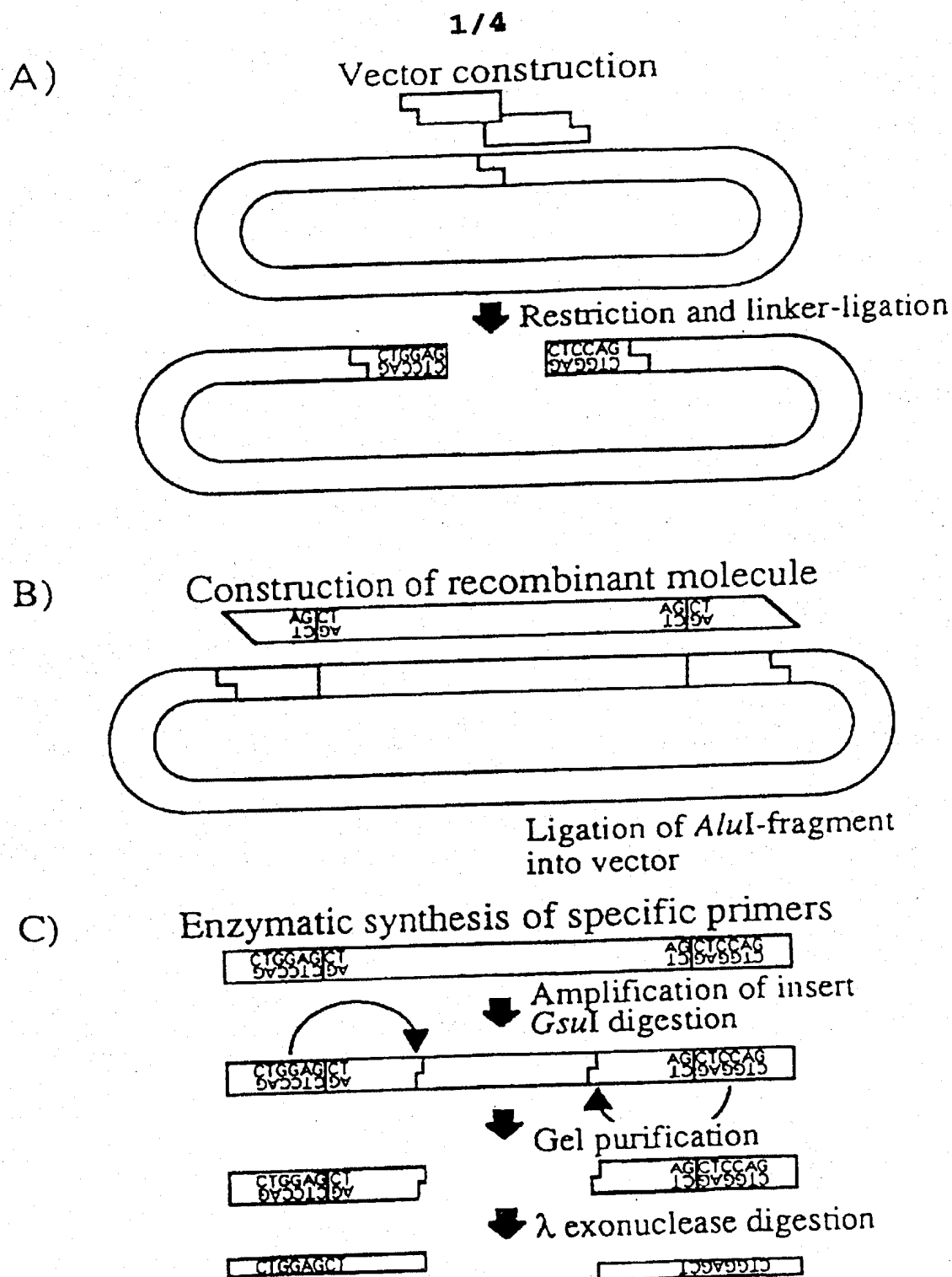
A) **Vector construction**

↓ Restriction and linker-ligation

B) **Construction of recombinant molecule**

Ligation of *Alu*I-fragment
into vector

C) **Enzymatic synthesis of specific primers**

↓ Amplification of insert
   *Gsu*I digestion

↓ Gel purification

↓ λ exonuclease digestion

FIG. 1A

SUBSTITUTE SHEET

D) Amplification using enzyme-synthesized primers
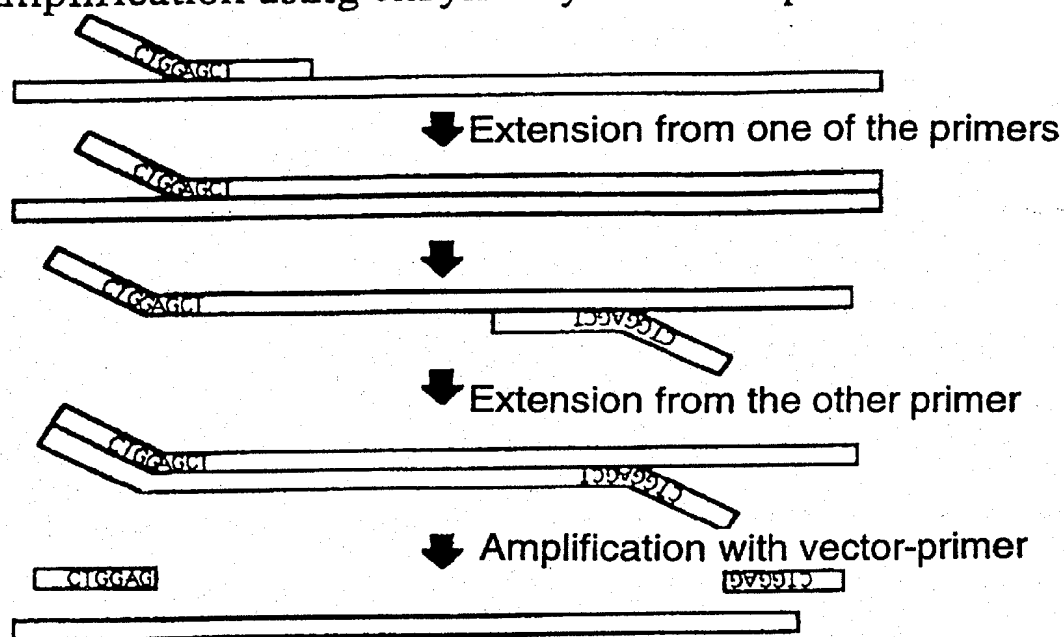
FIG. 1B
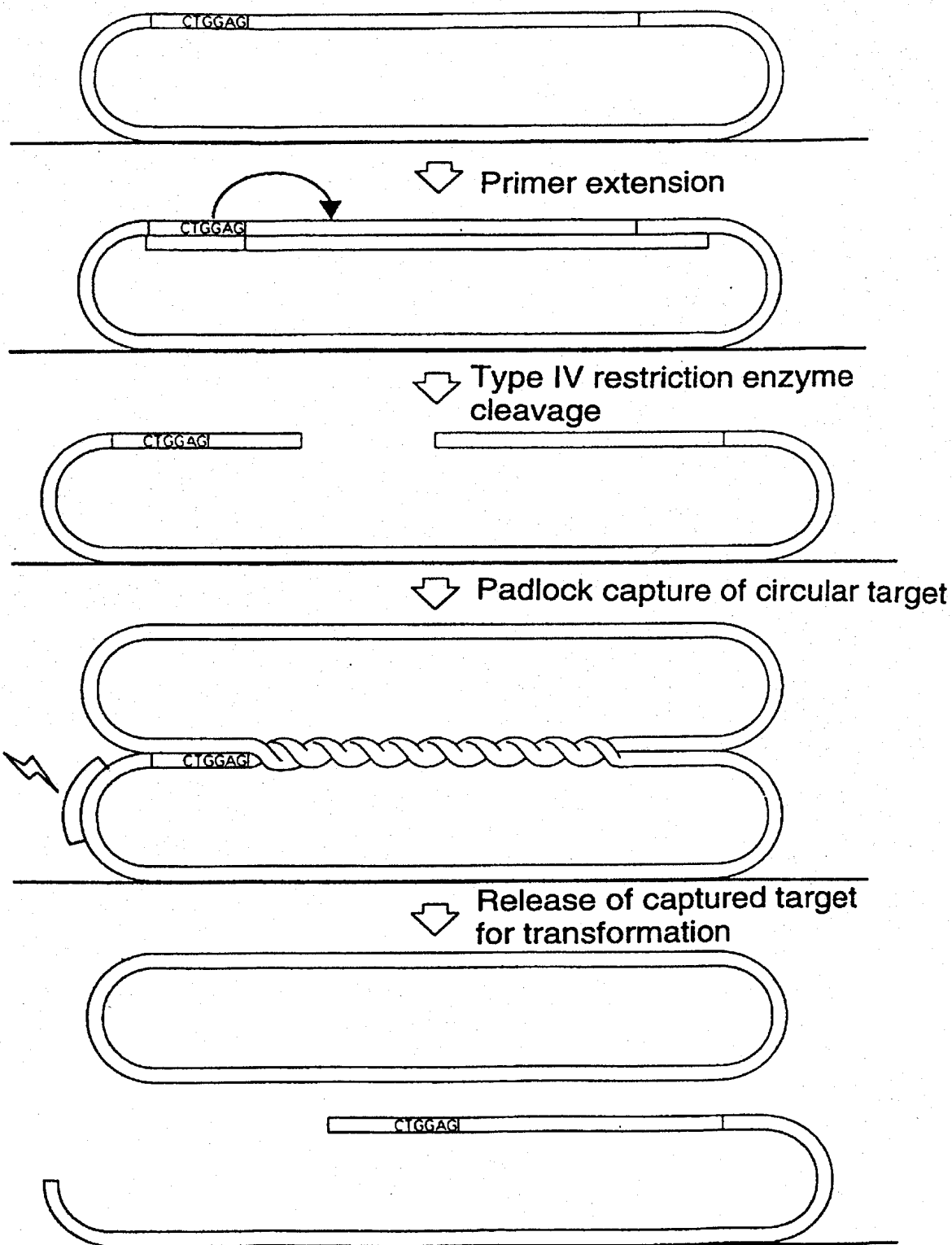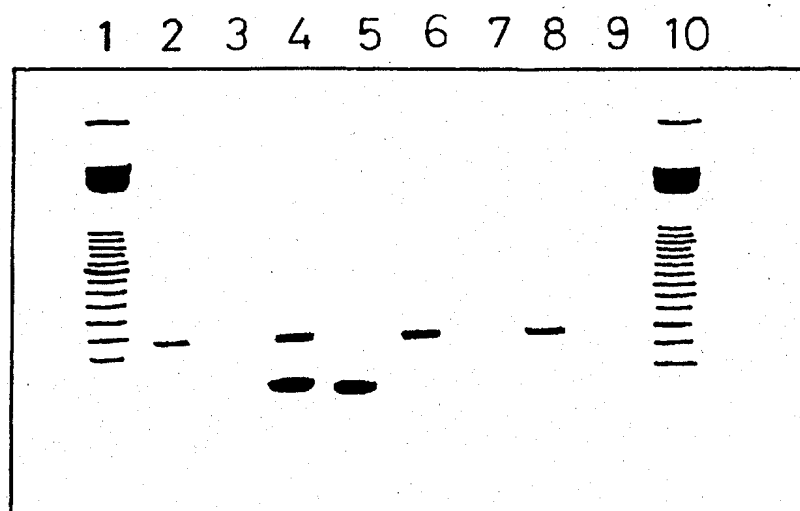
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$\Downarrow$ Primer extension

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$\Downarrow$ Type IV restriction enzyme cleavage

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$\Downarrow$ Padlock capture of circular target

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$\Downarrow$ Release of captured target for transformation

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FIG. 2

FIG. 3

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/10, C12N 15/66 // C12Q 1/68
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed classification symbols)

IPC6: C12N, C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EDOC, BIOSIS, MEDLINE, DBA, SCISEARCH, PATENT CITATION INDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GENE Volume 54, 1987, R.B. GAYLE III et al, ormation of MboII vectors and cassettes using asymmetric MboII linkers" page 221 - page 228	1-17
A	WO 9001548 A1 (CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED), 22 February 1990 (22.02.90)	1-17

☐ Further documents are listed in the continuation of Box C.   ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 March 1996	14.03.96
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9001548	22/02/90	NONE	